EVOLUTION OF ANTIBODY RESPONSE AND FUNGAL ANTIGENS IN THE SERUM OF A PATIENT INFECTED WITH *CANDIDA FAMATA*

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SUMMARY

The presence of both fungal antibodies and antigens in the serum of a patient with acute zonal occult outer retinopathy caused by *Candida famata* infection has been examined. Antibodies against *C. famata* increased until 1999-2000 when antifungal treatment was initiated. The antibodies were detected by ELISA and immunofluorescence analysis using *C. famata*. These antibodies were not immunoreactive against several candida species tested. Positive immunofluorescence was obtained with IgM, but not IgA, IgG or IgE. Moreover, the IgM response disappeared several months after treatment with antifungal compounds, despite the fact that *C. famata* antigens were present in blood. Finally, a sensitive test was developed to assay for the presence of *C. famata* antigens in serum. This method is based on the immunodetection of fungal antigens transferred to a nitrocellulose membrane that is incubated with rabbit antibodies rose against *C. famata*. According to this method, the infection remitted with antifungal treatment.

INTRODUCTION

The immunologic response to fungal infections in humans is complex and still subject to much debate (Coleman *et al.*, 1998; d'Ostiani *et al.*, 2000; Gil, 1997; Lopez-Ribot *et al.*, 2004; Mencacci, 1999; Pfaller, 1996; Shoham & Levitz, 2005). Cellular response, particularly macrophage function may be crucial to combat systemic infections produced by different fungi (Badauy *et al.*, 2005; d'Ostiani *et al.*, 2000; Simitsopoulou & Roilides, 2005; Villar *et al.*, 2005). However, the importance of a robust antibody response to candida infections has also been documented (Fernandez-Arenas *et al.*, 2004; Mencacci, 1999; Witkin *et al.*, 1983). The fact that systemic candidiasis is much more common in immunocompromised patients evidences the key role of the immune system in preventing this infection (Altamura *et al.*, 2001; Clemons *et al.*, 2000; Ruhnke, 2006; Sims *et al.*, 2005). Cellular immune response, together with the humoral response and innate immunity are most likely necessary for an efficient control of this type of systemic infection (Altamura *et al.*, 2001; Lopez-Ribot *et al.*, 2004). Although many studies have described the immune response in human candidiasis (Lopez-Ribot *et al.*, 2004; Mencacci, 1999; Rozell *et al.*, 2006), hardly any have attempted to characterize the antibodies of patients infected with *C. famata*.

Candida spp. accounts for a large percentage of human fungal infections. Apart from *Candida albicans*, which represents about 50% of blood isolates (Canteros *et al.*, 1994; Peres-Bota *et al.*, 2004), other species such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, have been found in the blood of infected patients (Al-Hedaithy, 2003; Almirante *et al.*, 2006; Brandt *et al.*, 2000; Fanci *et al.*, 2005; Veldman *et al.*, 2006; Wagner *et al.*, 2005; Yang *et al.*, 2003). Viable *C. famata* has been found in about 1-2% of patients suffering systemic candidiasis (Al-Hedaithy, 2003; Canteros *et al.*, 1994; Krcmery

& Barnes, 2002; Peres-Bota *et al.*, 2004; Pfaller *et al.*, 2003; Prinsloo *et al.*, 2003; Ruhnke, 2006; Tortorano *et al.*, 2006; Yamamoto *et al.*, 2002), but analysis of the presence of genomes by PCR yields a different percentage (Deventer, 1995; Khan & Mustafa, 2001; Pryce *et al.*, 2003). Although *C. famata* is considered non-pathogenic (Andrighetto *et al.*, 2000; Gardini *et al.*, 2001), it has been found in tissues as diverse as bone, blood or the CNS, and is associated with vision problems (Krcmery & Kunova, 2000; Prinsloo *et al.*, 2003; Rao *et al.*, 1991; St-Germain & Laverdiere, 1986). Recent evidence suggests that *C. famata* was the etiological agent responsible for acute zonal occult outer retinopathy (AZOOR). We now report in detail the antibody response in an AZOOR patient previously described (Carrasco *et al.*, 2005). We also monitored the course of infection using different methods. Correct diagnosis of disseminated candidiasis is still elusive in some patients; thus, different approaches are needed to demonstrate disseminated fungal infection (Ellepola & Morrison, 2005; Pontón, 2006; Yeo & Wong, 2002). In most hospitals, hemocultures are the main routine assay for detecting disseminated candidiasis (Ellepola & Morrison, 2005). However, unlike systemic candidiasis, viable candida cells are very seldom present in disseminated infections. PCR can overcome this problem but at least one fungal genome per assay must be present to determine the presence of yeast cells in blood (Bretagne & Costa, 2005). Other methods are based on detecting fungal metabolites or other components, like proteins or polysaccharides in blood serum (Ishibashi *et al.*, 2005; Mitsutake *et al.*, 1996; Pontón, 2006). Some of these methods detect specific proteins, such as glycolytic enzymes, heat-shock proteins or secreted proteases. The use of immunological assays to evidence the presence of these proteins in human blood serum constitutes a more sensitive test to diagnose disseminated candidiasis. Diverse commercial kits have been developed to assay the presence of polysaccharides such as manans or glucans in serum.

We compared all these techniques in serum samples taken at different times from a patient infected with *C. famata*. Our results suggest that analysis of fungal proteins in serum constitutes the most sensitive test to monitor the course of this infection.

METHODS

Yeast growth. The yeast was grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) by incubation at 30°C. The same medium, containing agar, was used to isolate individual yeast colonies.

Antibodies. Rabbit antisera against different yeast species were obtained by inoculation of 0.5 ml of phosphate buffered sorbitol (PBS) containing 1 or 2 mg of yeast after autoclaving and lyophilization. Each inoculum had been previously mixed with the same volume of Freund's adjuvant. Rabbits were inoculated up to four times, and the antibody titer and specificity of the sera were tested by immunofluorescence and Western blotting.

Immunofluorescence and immune-electron microscopy. For *C. famata*, 1 ml of culture was placed in 1.5 ml microcentrifuge tubes. Cells were washed with PBS, incubated with 50 mM ammonium chloride for 10 min, and washed three times with PBS-Tween 20. Cells were then treated with the different sera diluted 1:500 in PBS-Tween 20, at 37ºC for 2 h, washed again with PBS-Tween 20 and incubated with the secondary antibody. Goat anti-human immunoglobulins IgA, IgM and IgG (Sigma), or rabbit anti IgG+IgA+IgM (Abcam), conjugated to fluorescein antibodies, were added at 1:500 dilution; in some cases anti-IgG_4 and IgE antibodies were used (a generous gift from M. Lombardero, ALK, Abelló, Madrid). In these cases, samples were then incubated with an anti-Ig fluorescein-conjugated antibody. The samples were incubated at 37ºC for another hour. The cells were then washed, resuspended in PBS and mounted on slides with a drop of Depex (Serva). Finally, the cells were observed under a fluorescence microscope. For the remaining candida species, the commercial kit Euroimmun (Medizinische Labordiagnostika AG) was

used in accordance with the manufacturer's instructions and using the same serum dilutions as for *C. famata*. For immune electron microscopy, we followed the protocol described elsewhere (Wright *et al.*, 1988), with a few modifications: the cells were fixed for 2 h at room temperature in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and washed after postfixation with 0.15% tannic acid in 0.05 M cacodylate buffer (pH 7.2). Cells were finally embedded in Epon (TAAB laboratories, Berkshire, United Kingdom). The sections were rehydrated in PBS and immersed in 0.5 M NH_2Cl for 15 minutes. Samples were then blocked with 1% BSA in PBS, incubated for 1 hour with the primary antibody diluted in 1% low-fat dry milk in PBS at room temperature, and subsequently washed with 0.1% BSA in PBS. After washing, secondary antibody conjugated to colloidal gold particles was added at 1:10000 dilution in PBS. Finally, samples were washed again with PBS and once again with triply-distilled water. The samples were analyzed with a JEOL 1010 electron microscope.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was carried out as previously described (Carrasco *et al.*, 2005). Briefly, a *C. famata* cell suspension diluted in PBS was seeded in ELISA microtiter plates (Maxisorp; Nunc). Blockade was carried out in PBS containing 3% low-fat dry milk and 0.2% Tween 20. Sera from the AZOOR patient were added at 1:200 dilution and plates were subsequently incubated with goat anti-human (heavy and light chain) immunoglobulin G horseradish peroxidase-conjugated antibodies (Pierce) and washed five times. Color development was accomplished by incubation with *o*-phenylenediamine (Sigma) and measured at 490 nm in a microplate reader (EL340; BIO-TEK Instruments).

Western blot assays. Yeast proteins were precipitated with trichloroacetic acid (10%) and fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% polyacrylamide, transferred to nitrocellulose membranes by wet immunotransfer, and processed for Western blotting. Blocking, incubation with antibodies and washing were performed as described for ELISA. Goat anti-human IgM horseradish peroxidase-conjugated antibodies (Amersham Biosciences) and the ECL kit (Amersham) were used to detect bound antibodies. Chemiluminescence was detected by exposure to Agfa X-ray film.

PCR analyses. The DNA was extracted from serum or whole blood. To this end, 200 µl of serum were boiled for 10 minutes and then incubated for 2 h at 37ºC with Zimolase (ICN) and for a further 2 hours at 58ºC with proteinase K (Sigma). Then 200 µl of a detergent buffer were added and samples were boiled again for 10 minutes before adding 1 ml of phenol:chloroform (1:1) (Amersham) and centrifuging at 20000 × g for 20 min. The upper aqueous phase was recovered and washed twice with ethyl ether. The DNA was precipitated by addition of 3 volumes of absolute ethanol (Merck) (−20°C) to the aqueous phase. After storing the samples overnight at −20°C, the DNA was then centrifuged at 20000 × g for 20 min. Pellets were dried and resuspended in H_2O. The DNA preparations were incubated with oligonucleotides that hybridize to the rRNA genes (Li *et al.*, 2003; Nishikawa *et al.*, 1999). In this manner, the first ITS region was amplified. The amplified product was analyzed by agarose gel electrophoresis. The corresponding band was sequenced to determine unequivocally the presence of *C. famata* genomes. Real time quantitative PCR was carried out in an ABI PRISM 7000 thermocycler (*Applied biosystems*). The reaction mix was prepared with 0.9 µM of each oligonucleotide and 0.25 µM of TaqMan probe in a final volume of 20 µl, to which 50 ng of DNA were added. The

concentration of DNA template was normalized by previous PCR reactions with specific oligonucleotides, in which DNA was denatured at 95ºC for 10 minutes and amplified in 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. Analysis of the data was done using the *SDS 7000 (1.1)* software.

Dot-Blot analyses. 200 µl of different serum dilutions in TBS were added to each well. Samples were blotted onto a 45 mm nitrocellulose membrane (Bio-Rad) previously hydrated in TBS for 10 minutes using the Bio-Dot SF apparatus (Bio-Rad). After blotting, the membrane was processed and developed as described above for Western blotting. The primary antibodies, rabbit polyclonal antibodies rose against *C. famata*, *C. albicans*, *C. glabrata*, *C. parasilopsis*, *Rhodotorula mucilaginosa* or *Saccharomyces cerevisiae* were used at 1:1000 dilution. We used a donkey anti-rabbit IgG horseradish peroxidase-conjugated antibody (Amersham Biosciences) at 1:5000 dilution as secondary antibody.

Detection of fungal polysaccharides. The presence of manoses and beta-1,3 glucan in serum was detected using the Platelia Candida AG test (Bio-Rad), as described by the manufacturer, or the Fungitell Serum Assay kit, performed by Fontlab laboratories (Barcelona, Spain) (Pazos *et al.*, 2005).

RESULTS AND DISCUSSION

The characteristics of our patient have been been previously described (Carrasco *et al.*, 2005). Briefly, the patient was diagnosed with acute zonal occult outer retinopathy (AZOOR) in 1996 (aged 47). In 1999, *C. famata* was isolated from conjunctival exudate and blood. At the end of this year, intermittent antifungal treatment was administered as described (Carrasco *et al.*, 2005). Initially, the presence of anti-*C. famata* antibodies from serum obtained from different years was analyzed by ELISA (Figure 1A). The *C. famata* antibody titer increased until 1999. After administration of antifungal compounds, a clear decrease in the antibody titer was observed and it has remained low ever since. Analysis of these antibodies by immunofluorescence using *C. famata* also corroborated this observation (Figure 1B). Thus, positive immunofluorescence appeared with the sera obtained in June 1999, September 1999 and January 2000. Low reactivity was found with sera from June 1996 and August 2001, while no antibodies were detected from serum obtained in October 2005. The immunofluorescence observed with other yeast species was similar to the negative control without primary antibody. Only a slight reaction with *C. tropicalis* apperaed suggesting that the antibodies were fairly specific against *C. famata* in the immunofluorescence test. In our experience, some human sera do react with *C. tropicalis* in the Euroimmune test. Therefore, we consider this reactivity as non-specific. Next, we wanted to analyze the type of antibodies present in the positive serum from 1999. For this, a second anti-human antibody specific for IgA, IgM, IgG, IgG4 or IgE was employed after incubation of *C. famata* with this serum (Figure 2). Interestingly, this assay revealed the presence of IgM but not IgG antibodies, even though the patient had been infected over several years. On the other hand, these IgM antibodies were not cross-reactive with *C. albicans* (Figure 2). This serum was also analyzed by Western blotting against *C. famata*

proteins. Figure 3A shows that there are two yeast proteins that immunoreact better with serum from September 1999. These two proteins, of about 41 and 30 KDa, reacted more weakly with sera from October 2005, January 2006 and the control healthy donor. In addition, there are other proteins that immunoreact non-specifically with all the sera employed, such as the 47 KDa protein. Western blotting using *C. albicans* proteins reveals a low reactivity with the patient's serum, as compared to a serum from a control patient infected with *C. albicans*. Moreover, no immunoreactivity was found against proteins from *Cryptococcus magnus* (Figure 3A). Immuno-gold using electron microscopy analysis was carried out (Figure 3B). This human antiserum clearly reacted with antigens at or in close proximity to the plasma membrane of *C. famata*. For comparison, a rabbit antiserum rose against this yeast was employed. It was noteworthy that both human and rabbit antisera recognized a similar structure close to the plasma membrane.

Since the vision symptoms have not yet fully resolved, we tried to detect the presence of *C. famata* infection using PCR. DNA was extracted from serum submitted to PCR out using oligonucleotides that amplify the first intervening sequence between the rRNA genes, as described. Real-time quantitative PCR using TaqMan probes was carried out with different samples (Figure 4A). Evidence for the presence of *C. famata* genomes in the blood sera of this patient was found in the samples from October 1996 and June 1997, despite the fact that no viable yeast cells were recovered from these hemocultures. Further, the presence of fungal genomes clearly diminished from June 1999 even though some symptoms of infection remained in this patient, such as the appearance of *C. famata* genomes in conjunctival exudates. The decrease of fungal genomes in blood since October 1996 probably corresponded to the discontinuation of immunosuppression therapy from the end of that year. These findings indicate that the presence of *C. famata* can be evidenced by

PCR in samples that contain few antibodies and, conversely, no fungal genomes are found at the peak of the antibody response. To confirm that the fungal genomes amplified in figure 4A were from *C. famata*, the amplified product obtained from another PCR reaction was separated by agarose gel electrophoresis (Figure 4B). A single product of about 240 nt is apparent in the serum from June 1997, which corresponds to the same band obtained from *C. famata* DNA. This band was extracted and sequenced revealing that it indeed belonged to *C. famata*. Although no fungal genomes were detected by PCR from January 2000, abundant *C. famata* genomes were evidenced in a number of conjunctival exudates obtained up until May 2006 (data now shown).

Since no *C. famata* genomes were detected in sera from recent years, we aimed to develop a system to detect the presence of fungal antigens in the blood serum. Our attempts to find fungal proteins by Western blotting using a rabbit antiserum rose against *C. famata* were unsuccessful. A much more sensitive test is the detection of proteins using the dot-blot assay. In this test, different serum dilutions are transferred to a nitrocellulose membrane that is incubated with the rabbit anti-*C. famata* antibodies. Using this assay, the presence of *C. famata* antigens is evident (Figure 5A) and decreased over the years (Figure 5B). This test has the advantage that it can be quantitated by densitometry. Thus, with the 1/500 dilution, there is no immunoreactivity with sera from healthy donors (Figure 5A). On the other hand, the patient serum did not react with rabbit pre-immune serum. Notably, some serum samples from the patient gave high test values, particularly before antifungal treatment was initiated. Despite prolonged administration of antifungal compounds, there was still evidence of fungal antigens using the dot-blot assay. Voriconazole treatment was initiated at the beginning of March 2006 but, curiously, there was an increase in the yeast antigens found in the blood, perhaps reflecting the action of this antifungal compound in

tissues where infection persisted. For comparison, the same serum samples were tested for the presence of fungal polysaccharides (Ishibashi *et al.*, 2005). The Bio-Rad test Platelia Candida AG gave negative results in our samples (data not shown), whereas positive values were found when the Fungitell kit was employed. This last test, which measures β-1,3 glucans, indicated the presence of this polysaccharide in the patient's blood over several years, but with large variations in the different samples assayed. The patient had become negative for this test from January 2006 but tested positive with the dot-blot assay. By comparison, the dot-blot assay is much more sensitive than the Fungitell test (Matthews, 1998; Mitsutake *et al.*, 1996; Reboli, 1993), although both assays could be run to confirm the existence of fungal infection. Both assays could be useful for a first diagnosis, but the follow-up of the patient should be carried out with the dot-blot test. Serum from a patient may even test negative for both assays even though some residual infection remains in some tissues of the body.

Finally, the specificity of the dot-blot assay was analyzed employing several rabbit polyclonal antibodies rose against different yeast species (*C. albicans*, *C. parapsilosis*, *C. glabrata*, *Rhodotorula mucilaginosa*, and *Saccharomyces cerevisiae*). The potency and specificity of these antibodies were estimated using immunofluorescence and Western blot assays against the corresponding yeasts. Specific reactivity of the serum was exhibited against *C. famata* antibodies, whereas partial cross-reactivity was found with rabbit antibodies rose against other yeasts but not with the anti-*S. cerevisiae* antibodies (Figure 6). In conclusion, these findings indicate that patient serum contains antigens that are preferentially recognized by *C. famata* antibodies and that partially cross-react with antibodies against other related yeast species.

In this work, we have examined in detail one patient infected with *C. famata*, in order to generalize the findings reported, more patients should be studied. Little is known about the human immune response to disseminated infection by *C. famata*. In principle, this yeast has been classified as non-pathogenic and so does not produce an overt inflammatory reaction. *C. famata* may use different strategies to evade immune response, one of them being the production of superoxide dismutase that interferes with macrophage function (Garcia-Gonzalez & Ochoa, 1999). Other strategies common to other yeasts may be the production of a mucous environment, as well as precipitation of calcium carbonate at the sites of infection as a result of the drop in pH associated with fungal metabolic activity. In the patient studied here, the immune response as regards to antibody production was rather weak. The IgM response quickly disappeared after several months of antifungal treatment, even though the infection still remains in the periocular mucosa and probably in the retina and optic nerve. The cellular immune response of this patient measured by the presence of specific T-lymphocytes was also low, despite the fact that he was not immunocompromised and was healthy apart from the vision problems. The rather low stimulation of the immune system observed in this patient may thus be one of the reasons for the prolonged infection described. The stimulation of the immune system certainly may help to combat and eventually to eradicate this infection (Lopez-Ribot *et al.*, 2004).

From the point of view of diagnosis, we have developed and compared different approaches to detect *C. famata* infection. Although the presence of the antibody response should be assayed, like other candida infections, this test is of limited diagnostic value (Quindos *et al.*, 2004). It must be stressed that the presence of viable candida in blood was very rarely detected in our patient. Once again, although hemoculture should be done, the absence of yeast growth does not rule out the possibility of a systemic or disseminated

infection (Einsele *et al.*, 1997). The use of PCR is of greater value. Although this technique is very sensitive and we can detect a single genome copy present in the test tube in our assays, fungal genomes may not be circulating in the blood, i.e. the yeast may be located at focuses in different tissues without any cells, whether dead or alive, entering the bloodstream (Khan & Mustafa, 2001). Under these conditions, it is possible that the synthesis of polysaccharides may occur or export proteins could reach the blood and these could be detected in the serum (Mitsutake *et al.*, 1996; Pontón, 2006). In this regard, it is of special interest the system developed to analyze *C. famata* antigens by dot blot using a rabbit antiserum. This assay is very sensitive, since the presence of these antigens can be detected at 1/500 or even 1/1000 dilutions. With this test, we can monitor the efficacy of the different antifungal treatments. The amount of protein detected by this method is in the femtomolar range, which is below the limit of detection of Western blotting.

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FIGURE LEGENDS

Figure 1: (A) ELISA to determine the presence of antibodies against C. *famata* in patient sera from different years. The assay was carried out as described in Materials and Methods. (B) Immunofluorescence analysis of different yeasts using different patient sera. In the case of *C. famata*, the protocol described in Materials and Methods was followed. As positive control, rabbit antiserum against *C. famata* was employed; for the negative control, PBS instead of primary antibody was added. The commercial kit for immunofluorescence, Euroimmun, was used for the remaining yeast species analyzed. The controls were those provided by the commercial kit.

Figure 2: Antibody type examined by immunofluorescence with the serum from 1999. *C. famata* cells were treated as described in Materials and Methods and incubated with patient serum from June 1999. Then, a range of anti-human antibodies specific for different immunoglobulins were employed as secondary antibody. As a negative control, PBS, instead of primary antibody was added.

Figure 3: (A) Western blot of *C. famata* proteins. Different human sera were assayed as indicated in the Figure. The secondary antibody recognized IgM immunoglobulins. Serum dilutions were at 1/4000, whereas the secondary antibody was employed at 1/10000. (B) Immuno electron microscopy of *C. famata* cells. Yeast cells were incubated with patient serum or, as a control, with the rabbit serum obtained against *C. famata*, then incubated with the secondary antibody conjugated with colloidal gold.

Figure 4: Detection of *C. famata* genomes by PCR. (A) Determination of the number of *C. famata* genomes in 200 µl of serum by quantitative PCR, carried out as described in Materials and Methods. (B) PCR analysis of different serum samples obtained from the patient on the dates indicated above each lane. PCR assays were carried out with oligonucleotides that amplify a region of the rRNA internal transcribed spacer genes. Control 1: positive control of DNA extracted from *C. famata*; Control 2: negative PCR control without DNA; Control 3: negative control of DNA extraction of phosphate buffer. To the left of the gel the DNA markers in base units are indicated.

Figure 5: (A) Dot-blot detection of *C. famata* antigens in patient sera. Different dilutions of sera were blotted to a nitrocellulose membrane, which was incubated with the rabbit antiserum rose against *C. famata*. Rabbit pre-immune serum and sera from two healthy donors (control 1 and 2) were used as controls. (B) Densitometric values of the 1/500 dilution from the dot blot of *C. famata* antigen in different serum samples obtained from the patient between 1996 and 2006. (C) Detection of fungal polysaccharides in different serum samples using the Fungitell commercial kit. The discontinuous line indicates the minimum level of antigen necessary for the sample to be considered as positive. Control 1 and 2 are sera from healthy donors.

Figure 6: Dot-blot analysis of the patient serum using rabbit antibodies rose against different yeasts. Dilutions from 1:200 to 1:5000 of the serum obtained from the patient in June 1996 were blotted and the nitrocellulose membrane was incubated with different antisera rose against the indicated species. The rabbit antisera were obtained as described in Materials and Methods.